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Published in:
British Journal of Pharmacology

DOI:
[10.1111/bph.15409](https://doi.org/10.1111/bph.15409)

Publication date:
2021

Licence:
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Document Version
Publisher's PDF, also known as Version of record

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Singleton, S., Baptista-Hon, D. T., Edelsten, E., McCaughey, K. S., Camplisson, E., & Hales, T. G. (2021). TRV130 partial agonism and capacity to induce antinociceptive tolerance revealed through reducing available μ opioid receptor number. *British Journal of Pharmacology*, 178(8), 1855-1868. <https://doi.org/10.1111/bph.15409>

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RESEARCH PAPER

TRV130 partial agonism and capacity to induce anti-nociceptive tolerance revealed through reducing available μ -opioid receptor number

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Funding information

National Institute of Academic Anaesthesia

Background and Purpose: β -Arrestin2 recruitment to μ -receptors may contribute to the development of opioid side effects. This possibility led to the development of TRV130 and PZM21, opioids reportedly biased against β -arrestin2 recruitment in favour of G-protein signalling. However, low efficacy β -arrestin2 recruitment by TRV130 and PZM21 may simply reflect partial agonism overlooked due to overexpression of μ -receptors.

Experimental Approach: Efficacies and apparent potencies of DAMGO, morphine, PZM21 and TRV130 as stimulators of β -arrestin2 recruitment and inhibitors of cAMP accumulation were assessed in CHO cells stably expressing μ -receptors. Receptor availability was depleted through prior exposure of cells to the irreversible antagonist, β -FNA. We also examined whether μ -receptor availability influences TRV130 anti-nociception and/or tolerance using the tail withdrawal assay in wild-type C57BL/6 and μ +/- mice.

Key Results: Morphine, PZM21 and TRV130 were partial agonists in the β -arrestin2 recruitment assay. Only TRV130 exhibited partial agonism in the cAMP assay. Exposure to β -FNA to reduce μ -receptor availability further limited the efficacy of TRV130 and revealed morphine and PZM21 to be partial agonists. Despite having partial efficacy in vitro, TRV130 caused potent anti-nociception (ED_{50} : $0.33 \text{ mg} \cdot \text{kg}^{-1}$) in wild-type mice, without tolerance after daily administration for 10 days. TRV130 caused similar anti-nociception in μ +/- mice, with marked tolerance on day 4 of injections.

Conclusion and Implications: Our findings emphasise the importance of receptor reserve when characterising μ -receptor agonists. Reduced receptor availability reveals that TRV130 is a partial agonist capable of tolerance, despite having limited efficacy for β -arrestin2 recruitment to the μ -receptor.

Abbreviations: DAMGO, [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin; GIRK, G protein-coupled inwardly rectifying K⁺ channels; GRK, GPCR kinase; MPE, maximum possible effect; ORL-1, opioid receptor like-1; RLU, relative luminescence units; VACC, voltage-activated Ca²⁺ channel; WT, wild-type; β -FNA, β -funaltrexamine.

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KEYWORDS

arrestin recruitment, morphine, opioid analgesia, PZM21, receptor reserve, tolerance, TRV130

1 | INTRODUCTION

Opioids, such as the prototypical drug **morphine**, are currently the most effective analgesics combating short-term severe pain, but their chronic use is compromised by the development of side effects including tolerance, respiratory depression, constipation and hyperalgesia (Colvin et al., 2019). Additionally, opioid-induced euphoria and physical dependence contribute to their potential for addiction and diversion towards illicit use. The development of new analgesics that are devoid of detrimental side effects remains a challenge for adequate long-term pain management (Yekkirala et al., 2017).

Opioids activate **μ -receptors**, GPCRs that mediate their analgesic and their detrimental effects (Matthes et al., 1996). Activated μ -receptors signal through $G_{\alpha i/o}$ proteins to inhibit adenylyl cyclase activity and reduce cAMP accumulation. Activation of inwardly rectifying K^+ channels (**GIRK**) and inhibition of voltage-activated Ca^{2+} channels (**VACCs**) by the β subunits suppresses neuronal excitatory transmission leading to anti-nociception and analgesia (Heinke et al., 2011; Nockemann et al., 2013).

Activated μ -receptors also become phosphorylated by GPCR kinases (GRKs) leading to the recruitment of β -arrestin2. This initiates μ -receptor desensitisation, internalisation and recycling (Williams et al., 2013). Early evidence from knockout (*Arrb2*^{-/-}) mice implicated recruitment of β -arrestin2 in the development of opioid side effects including constipation, respiratory depression and tolerance (Bohn et al., 1999; Bohn et al., 2000; Raehal et al., 2005). While a subsequent study verified the need for β -arrestin2 for morphine tolerance (Bull, Baptista-Hon, Sneddon, et al., 2017), respiratory depression by morphine and **fentanyl** was recently observed in β -arrestin2 knockout mice (Kliwer et al., 2020). Furthermore, a conditional knock-in mouse model in which μ -receptors are deficient in phosphorylation by GRKs and therefore incapable of β -arrestin2 recruitment, exhibited morphine respiratory depression and constipation, with negligible tolerance (Kliwer et al., 2019). These findings confirm that β -arrestin2 recruitment to the μ -receptor is crucial for analgesic tolerance.

Evidence for the role of β -arrestin2 recruitment in the adverse effects of opioids led to the search for μ -receptor agonists biased in favour of G-protein signalling (Raehal et al., 2005). Recently, **PZM21** and **TRV130** (**oliceridine**), opioids reported to be biased towards G-protein activation with negligible β -arrestin2 recruitment were developed (Chen et al., 2013; Manglik et al., 2016). These agonists reportedly exhibit fewer side effects compared to morphine (DeWire et al., 2013; Manglik et al., 2016). However, recent evidence suggests that PZM21 and TRV130 are partial agonists (Yudin & Rohacs, 2019), a property that may account for the potentially superior side effect profiles of these analgesics (Gillis et al., 2020).

What is already known

- Partial agonism may account for the favourable side-effect profiles of reportedly biased μ agonists.

What this study adds

- Reducing receptor availability exposed partial agonism in cAMP assays and TRV130 anti-nociceptive tolerance *in vivo*.

What is the clinical significance

- TRV130 is capable of tolerance despite being a partial agonist.

Limited efficacy of PZM21 and TRV130 may have been overlooked in the original studies due to substantial receptor reserve in assays in which μ -receptors were overexpressed (Kelly, 2013). Partial agonists may display full efficacy in G protein-mediated inhibition of cAMP accumulation, in which there is amplification, while having negligible efficacy to recruit β -arrestin2, in which there is little or no amplification (Baptista-Hon et al., 2020).

In this study, we tested the hypothesis that partial agonism of reportedly biased agonists was previously overlooked in some studies due to receptor reserve. We determined the relative efficacies and apparent potencies of μ -receptor agonists **DAMGO**, morphine, TRV130 and PZM21 as stimulators of β -arrestin2 recruitment and inhibitors of cAMP accumulation in CHO cells with full or partial μ -receptor availability after exposure to the irreversible antagonist **β -funaltrexamine** (β -FNA). We also investigated whether TRV130, recently approved for clinical use (U.S. Food and Drug Administration, 2020), causes anti-nociceptive tolerance when administered to C57BL/6 mice and μ +/- mice that have a 50% reduction in μ -receptor expression (Matthes et al., 1996).

2 | METHODS

2.1 | Cell culture

PathHunter CHO cells (DiscoverX, UK; RRID:CVCL_KY70) stably overexpressing β -galactosidase tagged human μ -receptors and

β -arrestin2 were used in this study to assess cAMP accumulation and β -arrestin2 recruitment in the same cell line expressing similar numbers of receptors. Cells were maintained in complete media composed of 1:1 DMEM/Ham's F12 + GlutaMAX™ (Gibco, UK), supplemented with 10% FBS, penicillin (5,000 U·ml⁻¹), and streptomycin (5,000 µg·ml⁻¹). The selection agents geneticin (500 µg·ml⁻¹) and hygromycin B (250 µg·ml⁻¹) were added to maintain stable expression of μ -receptors and β -arrestin2 throughout. Cells were cultured at 37°C in 5% CO₂ and routinely subcultured when they reached 80% confluence.

2.2 | Transfection

Cells were plated in complete media for transfection in 35-mm culture dishes at a density of 1×10^6 cells per dish. The following day, 2 µg of pGloSensor-22F cDNA (Promega, UK) was transfected into each dish using Lipofectamine 2000 at a 1:2.5 ratio (Invitrogen, UK) and left for at least 16 h. Successful transfection was verified using a parallel dish transfected with 2-µg pEGFP cDNA under the same conditions and visualised using fluorescence microscopy.

2.3 | β -Arrestin2 recruitment

The PathHunter enzyme fragment complementation assay (DiscoverX, UK) was used to assess β -arrestin2 recruitment to μ -receptors according to the manufacturer's specification. CHO cells were suspended in Opti-MEM (Gibco) and seeded onto a 96 half-well plate at a density of 5×10^3 cells per well and left to settle overnight. Agonists were prepared in Opti-MEM (at 10× concentrations) and diluted into the wells. Cells were incubated with agonists for 90 min at 37°C. The DiscoverX chemiluminescence substrate solution was added to each well and incubated for 2 h at room temperature in the dark. Luminescence was measured on a GloMAX Navigator luminometer (Promega, UK) using a 1-s integration time.

2.4 | cAMP accumulation

In parallel with β -arrestin2 recruitment experiments, inhibition of forskolin-stimulated cAMP accumulation was measured in the PathHunter CHO cells. Cells transfected with pGloSensor-22F were suspended in complete media and seeded onto a 96 half-well plate at a density of 1.5×10^4 cells per well and left to settle overnight (according to the manufacturer's specification). Complete media were replaced with assay buffer containing HBSS supplemented with 20-mM HEPES, 3-mM luciferin (Promega, UK) and 30-µM forskolin (HelloBio, UK) at pH 7.4. Cells were left to incubate for 2 h at room temperature in the dark before control luminescence was measured on a GloMAX Navigator at 1-s integration time. Agonists (at 10× concentration) were prepared in assay buffer and diluted into the wells

and left to incubate for 30 min at room temperature in the dark. A second luminescence reading was obtained at the end of agonist incubation.

2.5 | Reducing available receptors using β -FNA

The number of available μ -receptors was reduced using the irreversible antagonist β -FNA to assess the efficacy and potency for β -arrestin2 recruitment and inhibition of cAMP accumulation. For β -arrestin2 recruitment, CHO cells were incubated with β -FNA (diluted in Opti-MEM) for 1 h at 37°C. β -FNA was removed and the cells were washed twice with Opti-MEM before agonist addition. For the cAMP accumulation assay, the method above was followed, with the exception that the assay buffer was replaced with one which contained β -FNA after 15 min and incubated for 1 h at 37°C. β -FNA was removed and cells were immediately washed with assay buffer and left to recover for 15 min at room temperature. The assay buffer was replaced again and incubated for a further 30 min before the control luminescence was measured. Agonist addition and subsequent measurements were performed as described above.

2.6 | Animals

Wild-type (WT) and μ +/- mice of both sexes weighing 19–29 g and maintained on the C57BL/6J background were used to assess TRV130 anti-nociception. All mice were aged between 8 and 16 weeks and housed in the University of Dundee School of Medicine Resource Unit. Heterozygous μ +/- mice were generated by breeding WT C57BL/6J (RRID:IMSR_JAX:000664) and μ -/- (RRID:IMSR_JAX:007559) mice as previously described (Bull, Baptista-Hon, Lambert, et al., 2017). Genotypes were confirmed at the end of the experiment using the automated genotyping service provided by Transnetyx (Cordova, USA). Groups were matched to age and sex, with equivalent numbers coming from each cage where possible. Mice were kept in a temperature-controlled room maintained at 19–21°C with a 12-h light-dark cycle and free access to food and water. Behavioural experiments took place in the light phase at the same time each day. Mice were handled and habituated to restraint for 3 days prior to starting behavioural experiments. Animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the animal welfare and ethical committee at the University of Dundee. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.7 | Materials

PathHunter CHO cells and proprietary assay buffer used for chemiluminescence were purchased from DiscoverX (California, USA).

Luciferin K⁺ salt and pGloSensor-22F cDNA were purchased from Promega (Southampton, U.K.). All cell culture and assay buffer reagents used originate from Gibco and were purchased from Thermo Fisher Scientific (Loughborough, U.K.). DAMGO ([D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin; HelloBio), morphine (Sigma, UK), PZM21 (Cambridge Bioscience, UK), TRV130 (Generon, UK) and β -FNA (Tocris, UK) were prepared as 10-mM stock solutions in either water (morphine) or DMSO (DAMGO, PZM21, TRV130 and β -FNA).

2.8 | Drug administration

TRV130 (25-mg·ml⁻¹ stock prepared in DMSO) was reconstituted daily in 0.9% normal saline to a final concentration of 1 mg·ml⁻¹ then filtered using a 0.2- μ m syringe. All injections were administered subcutaneous into the scruff of the neck and dosed according to individual body weights.

2.9 | Anti-nociception

TRV130 anti-nociception was assessed *in vivo* using a tail withdrawal assay as previously described (Bull, Baptista-Hon, Sneddon, et al., 2017). Each mouse had the distal third of their tail placed into hot (48°C) water and the latency to withdraw was measured prior to and 30 min after, TRV130. Tails were submerged up to a maximum of 15 s to prevent tissue damage. All latencies were recorded with genotype and drug injection blinded.

2.10 | Tolerance

Dose–response relationships were determined on Day 1 and Day 10 by administering each mouse with escalating (0.01–10 mg·kg⁻¹) doses of TRV130. Withdrawal latencies were measured as described above following administration of each cumulative dose. For repeated administration, TRV130 was administered once per day at a dose of 3.2 mg·kg⁻¹. This was determined to be the minimum dose required to cause all mice to reach the maximum cut-off latency, when assessed during the initial dose–response on Day 1. Tolerance was assessed in WT and μ +/- mice by comparing the maximum possible effect (MPE; see Section 2.11) of TRV130 on consecutive days of testing.

2.11 | Data analysis

Luminescence values derived from β -arrestin2 recruitment assays are expressed as a percentage of maximum luminescence (relative luminescence units [RLU]) produced on each plate. Luminescence values derived from cAMP accumulation assays are expressed as a percentage of forskolin-stimulated cAMP concentration before the addition of agonist. Luminescence values after exposure to β -FNA are

expressed as the percentage of the maximum luminescence produced in the presence of the agonist without prior exposure to β -FNA. Efficacy and potency data are compared to values evoked by DAMGO in all cases *in vitro*, with the exception of β -FNA concentration–response relationships, which are compared to the maximum control response of each agonist. Concentration–response relationships were plotted and fitted using GraphPad Prism to the four parameter logistics equation:

$$\text{Response} = \text{Minimum} + \frac{\text{Maximum} - \text{Minimum}}{\left(1 + 10^{((\text{LogEC}_{50} - [\text{Agonist}]) \times \text{Hill Slope})}\right)},$$

where apparent potency (EC₅₀ or IC₅₀), Hill slope and maximum and minimum parameters were derived. Each dataset, derived from luminescence values from individual plates, was fitted with the above logistics function. Efficacy was measured as the difference between minimum and maximum values of the fitted data. Potency data were transformed into pEC₅₀ or pIC₅₀ values using the log EC₅₀ or IC₅₀ multiplied by -1. Constraints to individual fits were set to minimum ≥ 0 for β -arrestin2 recruitment and for cAMP accumulation assays. One β -arrestin2 assay replicate was excluded from final analysis and presentation because the associated Hill slope values differed significantly from the other datasets. Averaged concentration–response relationships were also fitted with the logistics function and these are shown in the figures for illustrative purposes only.

TRV130 anti-nociception *in vivo* is presented as the percentage of MPE and determined using the equation:

$$\% \text{MPE} = \frac{\text{drug latency} - \text{baseline latency}}{15 \text{ s} - \text{baseline latency}} \times 100,$$

where baseline and drug latency represent the time to withdraw (s) in the absence and presence of TRV130 respectively. ED₅₀ values for each animal were derived from the logistics function of individual dose–response relationships fitted while constraining maximum to $\leq 100\%$. Averaged dose–response relationships were also fitted with the logistics function and these are shown in the figure for illustrative purposes only.

2.12 | Statistics

Analysed data were imported into GraphPad Prism software Version 8.4.1 (RRID:SCR_002798) for statistical analysis. The normality and homogeneity of variance for datasets were confirmed. Sphericity was also confirmed prior to performing a mixed ANOVA. Data satisfying parametric assumptions were compared using a one-way or mixed ANOVA, or *t* test as appropriate. Data not satisfying these assumptions, even after transformation, were compared using the Kruskal–Wallis test. Data derived from fitting individual experiments are presented as mean \pm SEM or with 95% confidence intervals (CIs).

Fits to the averaged data in the figures are purely for illustrative purposes and the derived values were not used for statistical comparisons. In all cases, group sizes are the number of independent values and statistical comparisons were only performed on these values where $n > 5$. Sample sizes in vivo were determined in G*Power using our previous data assessing morphine anti-nociception and tolerance in μ +/- mice (Bull, Baptista-Hon, Sneddon, et al., 2017). Given the lack of previous literature examining the sex dependent effects of TRV130 anti-nociception, these were examined separately in groups of male and female mice. Differences were considered statistically significant when $P < .05$. Pairwise comparisons were performed with Bonferroni or Dunn's correction applied. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). We have followed the recommendations set out in the *British Journal of Pharmacology* editorials where they are relevant.

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY <http://www.guidetopharmacology.org> and are permanently archived

in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | PZM21 and TRV130 evoke negligible β -arrestin2 recruitment to μ -receptors

We used PathHunter CHO cells stably overexpressing human μ -receptors to assess β -arrestin2 recruitment to μ -receptors. Concentration–response relationships for DAMGO, morphine, PZM21 and TRV130 are plotted in Figure 1a. Mean efficacy and potency values derived from individual replicates are shown in Table 1. Morphine, PZM21 and TRV130 were significantly less efficacious than DAMGO as stimulators of β -arrestin2 recruitment. In terms of potency, there were no significant differences between DAMGO, morphine, PZM21 or TRV130 as stimulators of β -arrestin2 recruitment.

We next investigated the ability of PZM21 or TRV130 to antagonise DAMGO-mediated β -arrestin2 recruitment, as would be expected if they are competing for the DAMGO binding site and acting as partial agonists. Concentration–response relationships of PZM21 and TRV130 in the presence of 3- μ M DAMGO are plotted in Figure 1b. Our data show that both PZM21 and TRV130

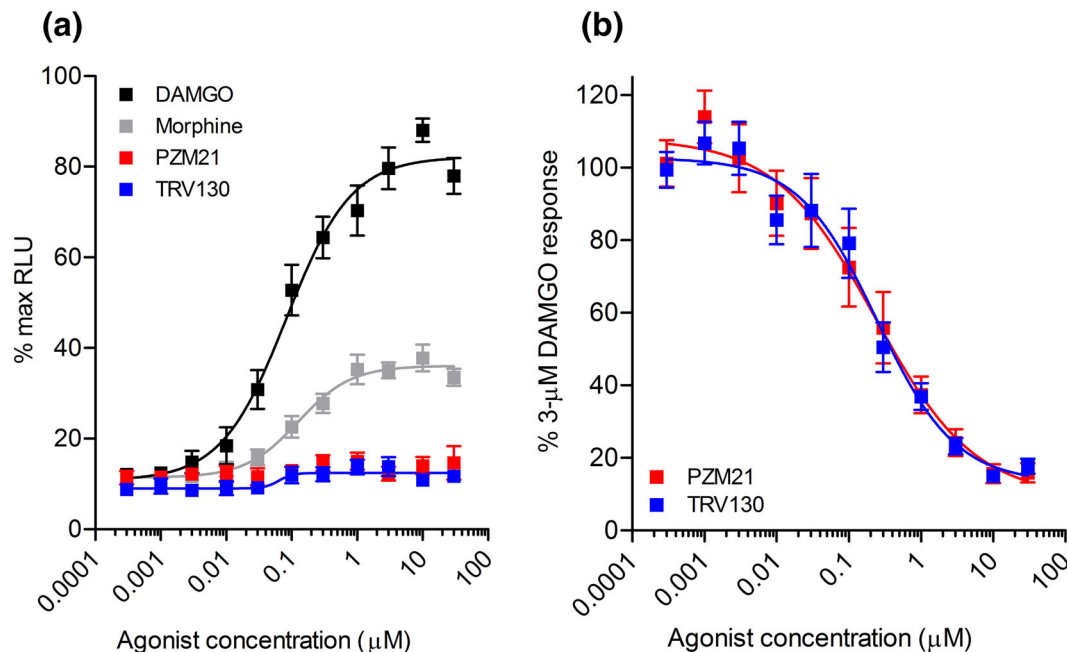


FIGURE 1 β -Arrestin2 recruitment to μ -receptors. (a) Concentration–response relationships of DAMGO, morphine, PZM21 and TRV130 for β -arrestin2 recruitment. Data from individual replicates were plotted and fitted with logistics functions to derive efficacy and potency parameters, presented in Table 1. Data are expressed as a percentage of maximum relative luminescence units (RLU) produced on each plate. All experiments were performed in duplicate with individual symbols showing mean \pm SEM, $n = 15$ (DAMGO), $n = 17$ (morphine), $n = 9$ (PZM21) and $n = 10$ (TRV130). One plate ($n = 1$) was excluded from data analysis and presentation (see Section 2). (b) DAMGO-evoked β -arrestin2 recruitment was inhibited in a concentration-dependent manner by either PZM21 or TRV130 co-application (0.0003–30 μ M). Data are expressed as a percentage of DAMGO (3 μ M)-evoked recruitment on the same plate. All experiments were performed in duplicate. Data are shown as mean \pm SEM, $n = 8$ for each agonist

Agonist	% max RLU	EC ₅₀ (nM)	pEC ₅₀	Hill slope	n
DAMGO	78 ± 3	208 (92, 325)	7 (6.6, 7.3)	1 ± 0.1	15
Morphine	26 ± 2*	195 (114, 276)	6.9 (6.6, 7.1)	1.4 ± 0.2	17
PZM21	4 ± 1*	183 (8, 359)	7.1 (6.6, 7.7)	1.7 ± 0.3	9
TRV130	5 ± 1*	139 (34, 244)	7.1 (6.7, 7.4)	1.6 ± 0.2	10

Note: Concentration–response relationships were plotted and fitted using a logistics function to yield efficacy and potency values as stimulators of β -arrestin2 recruitment. A one-way ANOVA detected a significant difference in mean efficacies ($F_{3,47} = 174.8$, $P < .05$). A one-way ANOVA revealed no significant difference in potencies ($F_{3,47} = 0.99$, $P > .05$). Efficacy and Hill slope values are presented as mean ± SEM and potency data are presented as mean with 95% confidence.

*Post hoc pairwise comparisons with Dunn's correction revealed morphine, PZM21 and TRV130 to be significantly less efficacious compared to DAMGO ($P < .05$).

TABLE 1 Efficacies and potencies of μ -receptor agonists as stimulators of β -arrestin2 recruitment

inhibited the ability of DAMGO to recruit β -arrestin2 in a concentration-dependent manner. A logistics function fitted to individual replicates revealed the mean IC₅₀ values (with CIs) of PZM21 and TRV130 to be 0.23 μ M (0.09, 0.59 μ M) and 0.25 μ M (0.13, 0.47 μ M), respectively, suggesting that both PZM21 and TRV130 bind to the same site as DAMGO but possess little efficacy to recruit β -arrestin2.

3.2 | TRV130 displays partial efficacy as an inhibitor of intracellular cAMP accumulation

We used PathHunter CHO cells, transiently transfected to express the GloSensor protein, to assess changes in intracellular cAMP accumulation as a consequence of μ -receptor activation. Concentration–response relationships of DAMGO, morphine, PZM21 and TRV130 are plotted in Figure 2. Mean efficacy and apparent potency values derived from individual replicates are shown in Table 2. The efficacy of TRV130 was significantly less than that of DAMGO, demonstrating that TRV130 is a partial agonist in the cAMP accumulation assay. Comparisons of the apparent potency values of these agonists also revealed morphine and PZM21 to be less potent than DAMGO, whereas the potency of TRV130 was similar to that of DAMGO.

3.3 | Stimulation of β -arrestin2 recruitment by DAMGO is more sensitive to depletion of available μ -receptors than inhibition of cAMP

A previous report (Spivak et al., 2003) suggests that, under certain conditions, β -FNA behaves as a partial μ agonist. Therefore, we assessed the ability of β -FNA to recruit β -arrestin2 or inhibit cAMP accumulation in CHO cells in the absence of agonists. CHO cells were exposed to β -FNA (up to 100 μ M) for 1 h and then unbound β -FNA was washed off, after which the protocols above for measuring β -arrestin2 recruitment or inhibition of cAMP accumulation were performed in the absence of agonist. β -FNA failed to recruit β -arrestin2

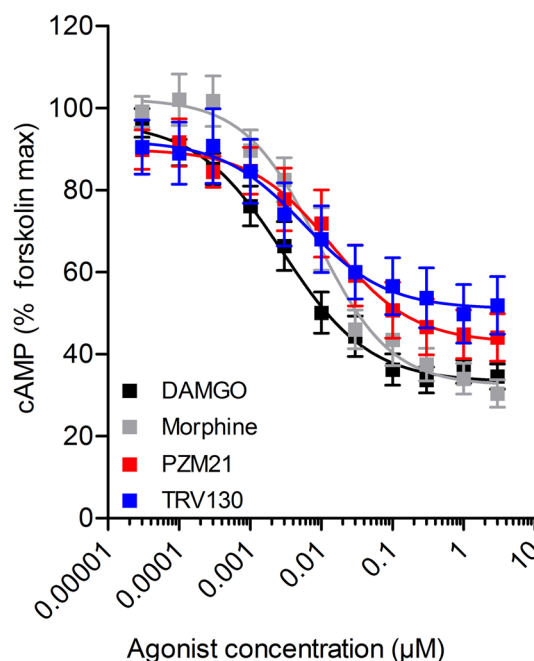


FIGURE 2 μ -Receptor activation inhibits cAMP accumulation. Concentration–response relationships of DAMGO, morphine, PZM21 and TRV130 as inhibitors of cAMP accumulation in PathHunter CHO cells transiently expressing the pGloSensor-22F protein. Data from individual replicates were plotted and fitted with logistics functions to derive efficacy and potency parameters, presented in Table 2. Data are expressed as a percentage of forskolin (30 μ M)-stimulated luminescence before the addition of agonists. All experiments were performed in duplicate. Data are shown as mean ± SEM, $n = 14$ (DAMGO), $n = 10$ (morphine) and $n = 9$ (PZM21 and TRV130)

($n = 4$) or elicit a change in intracellular cAMP accumulation ($n = 9$; data not shown).

We investigated the ability of DAMGO to recruit β -arrestin2 or inhibit cAMP accumulation, while the number of available μ -receptors was reduced following exposure to the irreversible μ -receptor antagonist, β -FNA. CHO cells were exposed to β -FNA for 1 h and then unbound β -FNA was washed off prior to the addition of agonists.

TABLE 2 Efficacies and potencies of μ -receptor agonists as inhibitors of cAMP accumulation following receptor depletion

Agonist	[β -FNA] (nM)	cAMP (% forskolin max)	IC ₅₀ (nM)	pIC ₅₀	Hill slope	n
DAMGO	0	64 ± 4	5 (1, 9)	8.5 (8.3, 8.8)	1 ± 0.1	14
	10	54 ± 4	14 (3, 24)	8 (7.6, 8.4) ^d	1.3 ± 0.3	6
	100	59 ± 9	112 (74, 150)	7 (6.8, 7.1) ^d	1 ± 0.1	8
Morphine	0	71 ± 4	13 (6, 21)	8 (7.7, 8.3) ^c	0.9 ± 0.1	10
	10	56 ± 2	69 (31, 107)	7.2 (7, 7.5) ^d	1.1 ± 0.2	6
	100	36 ± 4 ^b	225 (55, 395)	6.8 (6.4, 7.2) ^d	1.6 ± 0.3	8
PZM21	0	51 ± 4	16 (3, 29)	8 (7.6, 8.4) ^c	1 ± 0.2	9
	10	39 ± 5	41 (1, 85)	7.6 (7.1, 8)	1 ± 0.3	6
	100	36 ± 4 ^b	504 (33, 976)	6.5 (6.1, 6.8) ^d	0.9 ± 0.3	7
TRV130	0	45 ± 4 ^a	9 (1, 20)	8.4 (8, 8.8)	1 ± 0.2	9
	10	30 ± 4 ^b	52 (17, 87)	7.4 (7, 7.7) ^d	0.8 ± 0.2	6
	100	20 ± 2 ^b	141 (28, 254)	7 (6.7, 7.4) ^d	1.7 ± 0.5	7

Note: Concentration–response relationships were plotted and fitted using the logistics function to yield efficacy and potency values for μ agonists as inhibitors of cAMP accumulation with full (control) or partial μ -receptor availability following exposure to 10- or 100-nM β -FNA. A one-way ANOVA was used to compare mean efficacies ($F_{3,38} = 7.49$, $P < .05$). The efficacy of DAMGO was not significantly different from control ($F_{2,25} = 0.89$, $P > .05$). A one-way ANOVA was used to compare potencies ($F_{3,38} = 3.4$, $P < .05$). All experiments performed in duplicate. Efficacy and Hill slope presented as mean ± SEM and potency data are presented as mean with 95% confidence.

^aTRV130 had lower efficacy for cAMP inhibition compared to DAMGO without prior exposure to β -FNA ($P < .05$).

^bCompared to control (0 nM), exposure to 10-nM β -FNA reduced the efficacy of TRV130 to inhibit cAMP accumulation and exposure to 100-nM β -FNA reduced the efficacies of morphine, PZM21 and TRV130 ($P < .05$).

^cPost hoc pairwise comparisons with Dunn's correction revealed morphine and PZM21 to be less potent than DAMGO for cAMP inhibition without prior exposure to β -FNA ($P < .05$).

^dPairwise comparisons also revealed that, compared to control (0 nM), exposure to 10-nM β -FNA reduced the potency of DAMGO, morphine and TRV130 and exposure to 100-nM β -FNA reduced the potency of all agonists ($P < .05$).

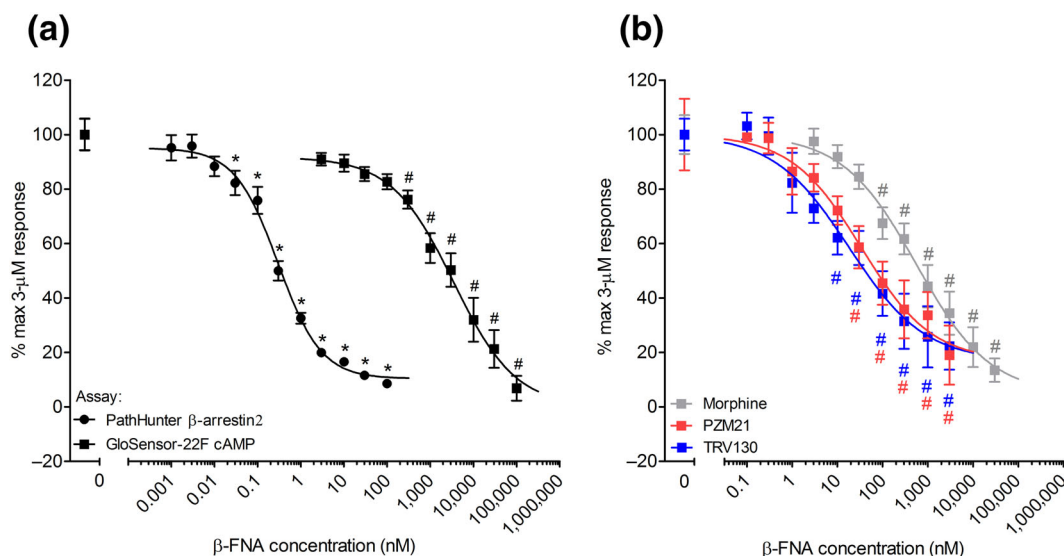


FIGURE 3 β -FNA causes irreversible, concentration-dependent antagonism of μ -receptor agonist-evoked signalling. (a) DAMGO-stimulated β -arrestin2 recruitment (circles) and cAMP inhibition (squares) following exposure to increasing concentrations of β -FNA. Data are expressed as a percentage of DAMGO (3 μ M) response. A one-way ANOVA was performed to determine the concentration of β -FNA that first caused a significant decrease in DAMGO-evoked response, in either the β -arrestin2 recruitment ($F_{11,115} = 76.02$, $P < .05$) or cAMP inhibition assay ($F_{11,119} = 51.14$, $P < .05$). Post hoc pairwise comparisons with Dunn's correction revealed the first significant decreases to occur at 0.03-nM β -FNA for β -arrestin2 recruitment ($^{\#}P < .05$) and 300-nM β -FNA for cAMP inhibition ($^{\#}P < .05$), $n = 13$ (β -arrestin2 recruitment) and $n = 11$ (cAMP accumulation). (b) The same approach was used to determine the concentration of β -FNA to cause the first significant decrease in 3- μ M morphine ($F_{11,115} = 45.97$, $P < .05$), PZM21 ($F_{14,119} = 11.96$, $P < .05$), or TRV130 ($F_{14,117} = 11.61$, $P < .05$)-evoked inhibition of cAMP accumulation. Post hoc pairwise comparisons with Dunn's correction revealed the first significant decreases to occur at 100 nM for morphine, 30 nM for PZM21 and 10 nM for TRV130 ($^{\#}P < .05$). All experiments performed in duplicate. Data fitted individually are shown as mean ± SEM, $n = 11$ (morphine) and $n = 12$ (PZM21 and TRV130)

Concentration–response relationships for β -FNA are plotted with a logistics function fitted to the data shown in Figure 3a. Data were expressed as the percentage of the maximum response produced by DAMGO (3 μ M) without prior exposure to β -FNA.

We determined the concentration of β -FNA required to produce the first significant reduction in the ability of DAMGO to recruit β -arrestin2 or inhibit cAMP accumulation using a one-way ANOVA across all concentrations tested. Post hoc pairwise comparisons with Dunn's correction revealed that the first significant reduction in the maximal DAMGO stimulation of β -arrestin2 recruitment occurred after exposure to 0.03-nM β -FNA. By contrast, the first significant reduction from maximal DAMGO-evoked inhibition of cAMP accumulation occurred after exposure to 300-nM β -FNA (Figure 3a). These data reveal a remarkable difference in the sensitivity to receptor availability of DAMGO's abilities to cause β -arrestin2 recruitment and inhibition of cAMP.

We used the same approach to determine the minimum concentration of β -FNA required to reduce the maximum inhibition of cAMP accumulation by morphine, PZM21 or TRV130. All agonists were applied at 3 μ M, which produces maximum inhibition of cAMP accumulation (Figure 2). Concentration–response relationships for β -FNA are shown in Figure 3b. Post hoc pairwise comparisons revealed the concentrations of β -FNA required to produce the first significant reduction in maximum cAMP inhibition to be: 100 nM for morphine, 30 nM for PZM21, and 10 nM for TRV130 (Figure 3b). These data are consistent with the rank order of efficacies for these partial agonists being morphine > PZM21 > TRV130.

3.4 | The potency of β -arrestin2 recruitment by DAMGO is unaffected by irreversible μ -receptor antagonism

We used the same approach to investigate the effect that μ -receptor depletion has on the concentration–response relationship for DAMGO to recruit β -arrestin2. The ability of DAMGO to stimulate β -arrestin2 recruitment is abolished following exposure to concentrations of β -FNA exceeding 10 nM in the β -arrestin2 recruitment assay.

Therefore, the number of available μ -receptors was reduced by exposing the cells to 0-, 1- or 10-nM β -FNA as described. DAMGO concentration–response relationships following exposure to these concentrations of β -FNA are plotted in Figure 4. Mean efficacy and potency values derived from individual replicates are shown in Table 3.

As expected, exposure to β -FNA reduced the efficacy of β -arrestin2 recruitment by DAMGO in a concentration-dependent manner. However, the potency of DAMGO to stimulate β -arrestin2 recruitment following exposure to either 1- or 10-nM β -FNA was not significantly different to the potency of DAMGO without prior exposure to β -FNA. These data confirm that depletion of μ -receptors in β -arrestin2 recruitment assays reduces the efficacy of DAMGO to recruit β -arrestin2, while having little effect on agonist potency.

3.5 | Partial agonism by morphine, PZM21 and TRV130 emphasised by reducing the availability of μ -receptors

Compared to the β -arrestin2 recruitment assay, exposure to higher concentrations of β -FNA is required to reduce agonist efficacy in cAMP accumulation assays. Therefore, we subsequently exposed PathHunter CHO cells to either 10- or 100-nM β -FNA, to investigate the effect that depletion of μ -receptor availability has on their concentration–response relationships in the cAMP accumulation assay. This approach allowed us to compare efficacies and apparent potency values with increasingly restricted receptor reserve. Concentration–response relationships of all four agonists are shown in Figure 5. Mean efficacy and apparent potency values derived from individual replicates are shown in Table 2.

We compared the efficacies of DAMGO, morphine, PZM21 and TRV130 to inhibit cAMP accumulation following exposure to 10- or 100-nM β -FNA, to their efficacies to inhibit cAMP accumulation without exposure to β -FNA. There was no significant difference detected in the efficacy of DAMGO to evoke changes in cAMP accumulation following exposure to either concentration of β -FNA. By contrast, the

[β -FNA] (nM)	% max RLU	EC ₅₀ (nM)	pEC ₅₀	Hill slope	n
0	75 \pm 2	275 (147, 402)	6.7 (6.4, 7)	1.3 \pm 0.1	9
1	42 \pm 5*	355 (164, 546)	6.6 (6.3, 6.8)	1.2 \pm 0.1	9
10	19 \pm 3*	263 (152, 374)	6.7 (6.4, 6.9)	1.7 \pm 0.3	9

Note: Concentration–response relationships were plotted and fitted using a logistics function to yield efficacy and potency values for DAMGO-evoked β -arrestin2 recruitment following exposure to increasing concentrations of β -FNA. A Kruskal–Wallis test was used to compare efficacies ($\chi^2_{(2)} = 19.13$, $P < .05$). A one-way ANOVA detected no significant difference in mean potency values ($F_{2,24} = 0.36$, $P > .05$). Efficacy and Hill slope values are presented as mean \pm SEM and potency data are presented as mean with 95% confidence.

*Post hoc pairwise comparisons with Dunn's correction revealed DAMGO to be significantly less efficacious as a stimulator of β -arrestin2 recruitment following exposure to 1- or 10-nM β -FNA compared to DAMGO without prior exposure ($P < .05$).

TABLE 3 DAMGO-evoked β -arrestin2 recruitment following irreversible μ antagonism

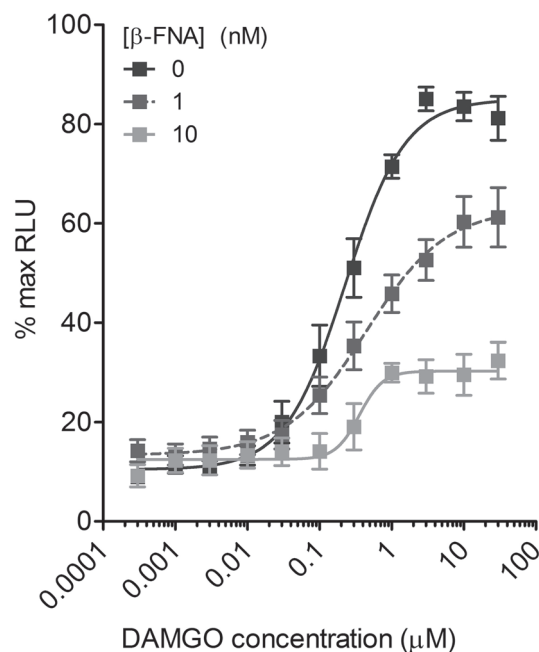


FIGURE 4 DAMGO-evoked β -arrestin2 recruitment following exposure to β -FNA. Concentration-response relationships of DAMGO-evoked β -arrestin2 recruitment in PathHunter CHO cells following exposure to 0-, 1-, or 10-nM β -FNA. Data from individual replicates were plotted and fitted with logistics functions to derive efficacy and potency parameters, presented in Table 3. Data are expressed as a percentage of maximum relative luminescence units (RLU) produced on each plate. All experiments performed in duplicate. Data are shown as mean \pm SEM, $n = 9$ per β -FNA concentration

efficacies of morphine, PZM21 and TRV130 were reduced following exposure to 100-nM β -FNA. The efficacy of TRV130 was also reduced following exposure to 10-nM β -FNA. These results confirm that morphine, PZM21 and TRV130 are partial μ -receptor agonists.

3.6 | TRV130 produces potent anti-nociception without evidence of tolerance in WT mice

We assessed TRV130 anti-nociception in vivo using a hot (48°C) water tail withdrawal assay in male and female C57Bl/6 mice. Opioid-naïve mice were exposed to escalating doses of TRV130 (ranging from 0.01 to 10 mg·kg⁻¹) and the latencies to withdraw their tails were measured and expressed as maximum possible effect (MPE, see Section 2). Dose-response relationships of TRV130 in WT mice are shown in Figure 6. Data are shown pooled for males and females after determining that there was no significant difference in the potency of TRV130 anti-nociception between sexes in WT mice (unpaired Student's *t*-test). TRV130 caused potent anti-nociception with a mean ED₅₀ of 0.33 (0.21, 0.46) mg·kg⁻¹ when assessed on Day 1. Furthermore, TRV130 caused the maximum possible level of anti-nociception in all mice at a cumulative dose of 3.2 mg·kg⁻¹ (Figure 6a).

After establishing that TRV130 evokes the maximal anti-nociceptive effect in WT mice at a cumulative dose of 3.2 mg·kg⁻¹, we then investigated whether tolerance occurred in the same mice exposed daily to this dose for 10 days. We observed a small rightward shift in the maximum possible effect of TRV130 after 10 days (Figure 6a). However, TRV130 was equally potent on Day 10 with a mean ED₅₀ of 0.68 (0.23, 1.13) mg·kg⁻¹ (Figure 6b). Furthermore, the baseline latency on Day 10 was not significantly different compared to Day 1. These results confirm that the lack of observed tolerance in WT mice is not due to altered baseline latencies.

3.7 | Reduced μ -receptor availability caused TRV130 anti-nociceptive tolerance

We similarly assessed TRV130 anti-nociception in μ +/- mice, which express 50% fewer receptors than WT mice (Matthes et al., 1996).

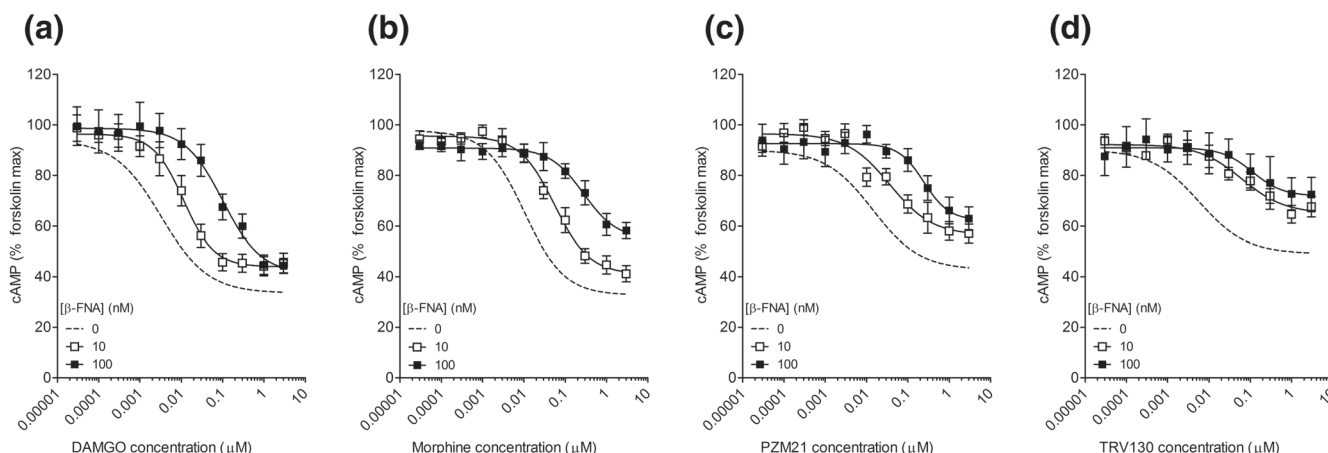


FIGURE 5 Partial agonism of morphine, PZM21 and TRV130 emphasised by reducing the availability of μ -receptors. Concentration-response relationships of (a) DAMGO, (b) morphine, (c) PZM21 and (d) TRV130 to inhibit cAMP accumulation following exposure to 10- or 100-nM β -FNA. Data from individual replicates were plotted and fitted with logistics functions to derive efficacy and potency parameters, shown in Table 2. Concentration-response relationships in the absence of β -FNA are reproduced for comparison (dashed black lines)

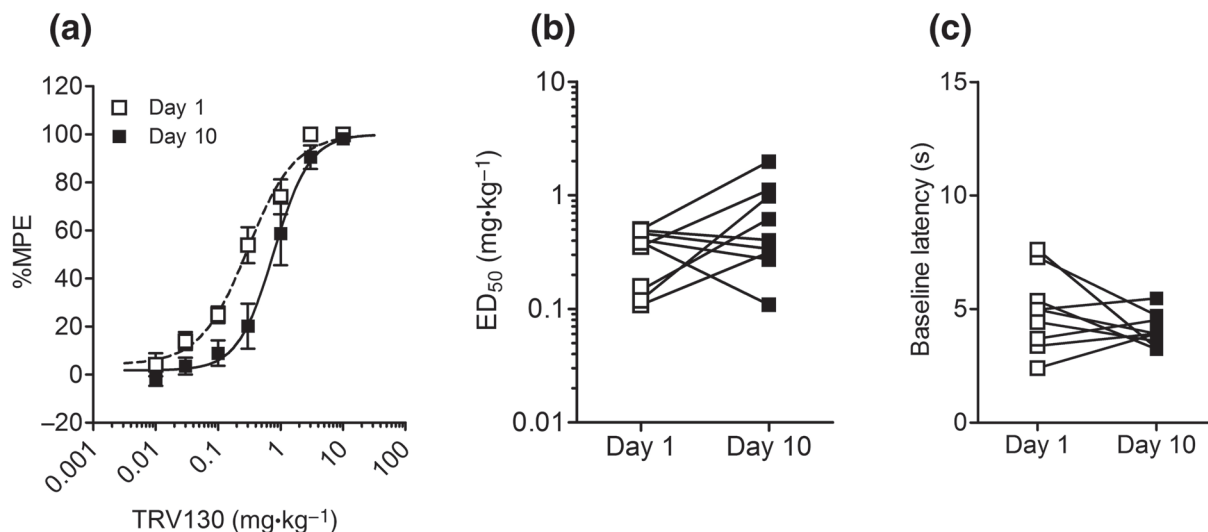


FIGURE 6 TRV130 causes potent anti-nociception without evidence of tolerance in C57Bl/6 mice. (a) Cumulative dose–response relationships for TRV130 anti-nociception in WT mice administered with TRV130 acutely (Day 1) or following 10 consecutive days (3.2 mg·kg⁻¹·day⁻¹). Data are expressed as the per cent maximum possible effect (MPE; see Section 2). (b) ED₅₀ values on Day 1 (0.33 (0.21, 0.46) mg·kg⁻¹) were not significantly different to Day 10 (0.68 (0.23, 1.13) mg·kg⁻¹; $P > .05$, paired Student's *t*-test). (c) Baseline latencies on Days 1 and 10 were not significantly different ($P > .05$, paired Student's *t*-test). Dose–response relationships from individual animals were plotted and fitted with logistics functions to derive TRV130 potency. Data are shown as mean \pm SEM, $n = 9$

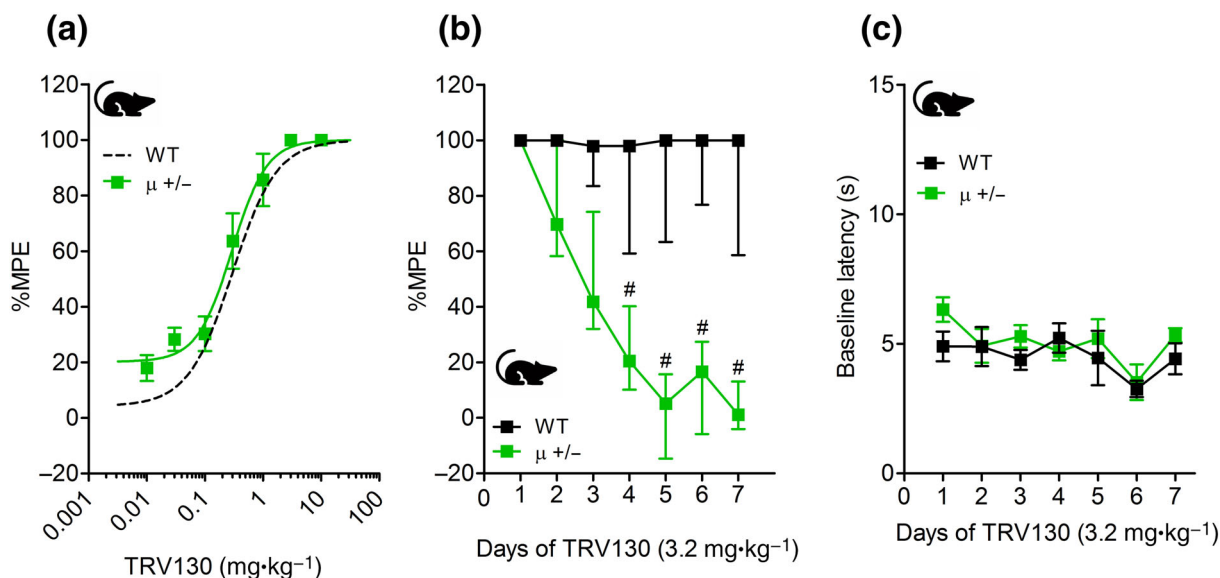


FIGURE 7 Reduced μ -receptor number causes the appearance of TRV130 anti-nociceptive tolerance. (a) Cumulative dose–response relationship for TRV130 anti-nociception in C57Bl/6 mice expressing 50% fewer μ -receptors (μ ^{+/-}) than WT mice. ED₅₀ values in μ ^{+/-} mice (0.44 (0.1, 0.84) mg·kg⁻¹), derived from logistics functions fitted to individual dose–response relationships, were not significantly different compared to WT mice ($P > .05$, unpaired *t* test with Welch's correction). The logistic fit to the mean TRV130 dose–response relationship for WT mice is reproduced for comparison. (b) TRV130 anti-nociception in WT and μ ^{+/-} mice receiving a 3.2-mg·kg⁻¹ dose for seven consecutive days. A Kruskal–Wallis test comparing MPE in WT versus μ ^{+/-} mice detected a significant difference ($\chi^2_{(13)} = 98.12$, $P < .05$). Post hoc pairwise comparisons with Dunn's correction revealed μ ^{+/-} mice had significantly shorter withdrawal latencies than WT mice after 4 days (* $P < .05$). (c) Baseline withdrawal latencies in WT and μ ^{+/-} mice across seven consecutive days of behavioural testing. A mixed-model ANOVA comparing baseline latencies revealed no significant effect of genotype ($F_{1,17} = 1.99$, $P > .05$) or time ($F_{6,102} = 0.64$, $P > .05$). Data in (a) and (c) are shown as mean \pm SEM. Data in (b) are presented as median \pm IQR, $n = 9$ (WT) and $n = 10$ (μ ^{+/-})

Dose–response relationships are shown in Figure 7. Data are pooled for males and females after finding that there was no significant difference in the potency of TRV130 anti-nociception between sexes in

μ ^{+/-} mice (unpaired *t* test). TRV130 was similarly potent in μ ^{+/-} mice with a mean ED₅₀ of 0.44 (0.1, 0.84) mg·kg⁻¹. There was no significant difference in potency between WT and μ ^{+/-} mice following

acute exposure (Figure 7a). Furthermore, the maximum level of TRV130 anti-nociception was also produced in all μ +/- mice following a cumulative dose of $3.2 \text{ mg}\cdot\text{kg}^{-1}$.

We investigated the effect that reduced μ -receptor number had on the development of anti-nociceptive tolerance to daily TRV130. WT and μ +/- mice had their withdrawal latencies measured as described above both before and 30 min after TRV130 ($3.2 \text{ mg}\cdot\text{kg}^{-1}$). Maximum possible effect in WT and μ +/- mice repeated over seven consecutive days is shown in Figure 7b. Pairwise comparisons with the Bonferroni correction revealed that μ +/- mice had significantly lower maximum possible effect compared to WT mice, after 4 days of daily TRV130 administration. Our results demonstrate that reduced availability of μ -receptors in vivo causes the appearance of anti-nociceptive tolerance to TRV130.

Baseline withdrawal latencies in WT and μ +/- mice across seven consecutive days of behavioural testing are shown in Figure 7c. No significant effect of genotype was detected in baseline withdrawal latencies between WT and μ +/- mice. These results confirm that the reduction in maximum possible effect observed in μ +/- mice is not due to altered baseline latencies.

4 | DISCUSSION

The results presented here confirm previous studies (DeWire et al., 2013; Manglik et al., 2016) demonstrating that PZM21 and TRV130 cause limited stimulation of β -arrestin2 recruitment to human μ -receptors, while having higher efficacies as inhibitors of cAMP accumulation. Importantly, our initial cAMP and β -arrestin2 recruitment assays were performed in cells with the same μ -receptor density. Under these conditions, TRV130, unlike the other agonists tested, lacked full efficacy as an inhibitor of cAMP. However, when μ -receptor availability was reduced by prior exposure to the irreversible μ -receptor antagonist β -FNA, morphine and PZM21 were also revealed to be partial agonists in the cAMP assay with lower efficacies than DAMGO. These findings suggest that partial agonism of reportedly biased agonists was previously overlooked in some studies due to receptor reserve.

Reduced receptor availability also decreased the apparent potencies of all agonists as inhibitors of cAMP accumulation. By contrast, reduced μ -receptor availability did not affect the potency of DAMGO as a stimulator of β -arrestin2 recruitment, despite reduced efficacy. The rank orders of the agonists' efficacies in the β -arrestin2 recruitment and cAMP assays (the latter with reduced receptor availability) were DAMGO > morphine > PZM21 = TRV130 and DAMGO > morphine = PZM21 > TRV130 respectively.

Negligible recruitment of β -arrestin2 to human μ -receptors by PZM21 and TRV130 may confer these agonists with favourable therapeutic properties. However, the benefits of poor β -arrestin2 recruitment are the subject of debate. Recent evidence (Hill et al., 2018; Kliewer et al., 2019; Kliewer et al., 2020) casts doubt on the interpretation of an earlier study linking β -arrestin2 to opioid-induced respiratory depression and constipation (Raehal et al., 2005). By contrast,

morphine anti-nociceptive tolerance is consistently found to be dependent on β -arrestin2 recruitment (Bohn et al., 2000; Bull, Baptista-Hon, Sneddon, et al., 2017; Kliewer et al., 2019; Yang et al., 2011). It is not surprising then that TRV130, which causes little recruitment of β -arrestin2 in vitro, caused no anti-nociceptive tolerance when administered daily to WT mice for 10 days, a finding consistent with previous reports (Altarifi et al., 2017; Liang et al., 2019). However, when administered under the same conditions to μ +/- mice, with 50% fewer receptors than WT mice (Matthes et al., 1996), TRV130 caused marked anti-nociceptive tolerance after only 4 days. We previously demonstrated that fewer available receptors also led to more rapid and profound morphine analgesic tolerance (Bull, Baptista-Hon, Sneddon, et al., 2017). The lack of tolerance to TRV130 in WT mice is likely due to the agonist's lower intrinsic efficacy, which limits β -arrestin2 recruitment and downstream effects such as receptor endocytosis. This, combined with substantial μ -receptor reserve, enables robust anti-nociception without tolerance in WT mice. Receptor reserve is evidenced by the fact that morphine and TRV130 also cause full anti-nociception in μ +/- mice, despite these ligands being partial μ -receptor agonists. It is important to note, however, that the use of moderate heat and an exposure limit of 15 s in our study may contribute to the appearance of full TRV130 efficacy in both WT and μ +/- mice (Smith et al., 1999).

We had anticipated that, given its low efficacy in the β -arrestin2 recruitment assay, TRV130 would not exhibit anti-nociceptive tolerance and in keeping with previous studies (Altarifi et al., 2017; Liang et al., 2019) this was true in the case of WT mice. However, robust tolerance occurred in μ +/- mice. Perhaps limited TRV130-evoked β -arrestin2 recruitment has a greater impact in μ +/- mice. However, our findings with β -FNA demonstrate that even with the high-efficacy agonist DAMGO, small reductions in μ -receptor availability cause large reductions in β -arrestin2 recruitment. The observation that TRV130 caused marked anti-nociceptive tolerance in μ +/- mice was therefore unexpected. It was also surprising that tolerance to TRV130 was similar to that observed previously with morphine in μ +/- mice (Bull, Baptista-Hon, Sneddon, et al., 2017), considering TRV130's lower efficacy than morphine in vitro.

It is hard to reconcile the finding that relatively small reductions in receptor availability impede β -arrestin2 recruitment in the cellular assay, while a large reduction in receptor availability in vivo enhances tolerance. This implies that there is a lack of a simple correlation between β -arrestin2 recruitment and tolerance and may reduce the likelihood of developing biased agonists that do not cause analgesic tolerance.

The situation in vivo is of course more complex than in the in vitro studies in which recombinant μ -receptors are overexpressed in relatively simple cellular models. In particular, neurones in the pain pathway and elsewhere in the brain often express more than one type of opioid receptor. This may affect μ -receptor activity either through the formation of heteromers or through signalling crosstalk (Charles et al., 2003; Fujita et al., 2015). When expressed together, μ - and δ -receptors recruit β -arrestin2 in the absence of agonist and this may alter the pattern of agonist-evoked activation of kinases and

agonist-evoked desensitisation (Bull, Baptista-Hon, Lambert, et al., 2017; Rozenfeld & Devi, 2007). Indeed, mice lacking δ -receptors exhibit negligible morphine anti-nociceptive tolerance (Zhu et al., 1999). Furthermore, most μ -receptor agonists have activity at other opioid receptors. For example, TRV130 is also a potent agonist at δ - and κ -receptors (Gutridge et al., 2020; Manglik et al., 2016). Therefore, other opioid receptors may contribute to TRV130 anti-nociception and tolerance. Our finding that potency is similar in μ +/- and WT mice is consistent with the idea that other receptors might contribute to TRV130 anti-nociception.

It is important to consider additional limitations of the in vitro assays used to compare G-protein signalling and β -arrestin2 recruitment, which may also compromise the characterisation of opioids and, in particular, estimates of agonist bias. In our study, both assays were performed in the same cells stably expressing μ -receptors and were therefore not unintentionally affected by differences in receptor reserve, which might otherwise confound comparisons of agonist efficacies. Nevertheless, it is clear that reductions in μ -receptor availability had profoundly different impacts on the two assays. Much more substantial β -FNA-evoked reductions in μ -receptor availability were required to impair the efficacy of DAMGO as an inhibitor of cAMP accumulation when compared to DAMGO's stimulation of β -arrestin2 recruitment. This is consistent with the idea that signalling to AC requires a small fraction of the available μ -receptors compared to recruitment of β -arrestin2, due to amplification in the former but not the latter (Baptista-Hon et al., 2020). The cAMP assay provides a highly sensitive method for the detection of the weakest agonists, while the β -arrestin2 recruitment assay yields a more accurate reflection of the relative potencies and efficacies of agonists. While it is experimentally expeditious to use simple cellular models for the identification of new analgesic opioids, more comprehensive approaches are needed for complete characterisation.

Interestingly, reductions in μ -receptor availability, insufficient to depress DAMGO's efficacy in the cAMP assay, were associated with decreased apparent potency. This was not the case in the β -arrestin2 recruitment assay in which DAMGO's efficacy declined without altered potency. This is likely because signalling to AC does not represent a simple 1:1 stoichiometry between receptor and effector. In this case, there is a contribution of agonist efficacy in the measurement of apparent potency (Colquhoun, 1998).

Numerous studies in recent years have focused on the potential for biased signalling in the development of new analgesic opioids including PZM21 and TRV130. However, our study supports previous reports indicating that these new opioids are in fact partial μ -receptor agonists, a property that potentially explains their favourable side-effect profile (Gillis et al., 2020; Hill et al., 2018). While these new opioids might prove to be useful, it is unclear how they will be superior as analgesics to the existing μ -receptor partial agonist **buprenorphine**. Buprenorphine has additional potentially beneficial antagonist and agonist activities at κ - and **opioid receptor like-1 (ORL-1) receptors**, respectively (Lutfy & Cowan, 2004). There may also be a contribution of a

longer μ -receptor residence time for buprenorphine than TRV130 (Pedersen et al., 2020) and differences in the pharmacokinetic profiles of the two opioids.

Our findings emphasise the need for consideration of receptor availability when characterising μ -receptor agonists and important insights that can be gained by taking measures to deplete receptor reserve. We suggest that it should become common practice to reduce receptor reserve during agonist characterisation to establish the true efficacies of new ligands and, in particular, before assignment of agonist bias.

ACKNOWLEDGEMENTS

We are grateful for helpful advice received during the early stages of this study from Professor Eamonn Kelly (University of Bristol) and Dr Chris Bailey (University of Bath). This study was supported by the National Institute of Academic Anaesthesia (NIAA), BJA Studentship.

AUTHOR CONTRIBUTIONS

S.S., D.T.B.-H., E.E., K.S.M. and E.C. performed the assays and, with input from T.G.H., analysed the data. S.S., D.T.B.-H. and T.G.H. conceived the study. S.S., D.T.B.-H. and T.G.H. wrote the paper.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design & Analysis](#) and [Animal Experimentation](#) and as recommended by funding agencies, publishers and other organisation engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Singleton S, Baptista-Hon DT, Edelsten E, McCaughey KS, Camplisson E, Hales TG. TRV130 partial agonism and capacity to induce anti-nociceptive tolerance revealed through reducing available μ -opioid receptor number. *Br J Pharmacol*. 2021;1–14. <https://doi.org/10.1111/bph.15409>